Comparison of spectrophotometric estimates of chlorophylls-*a*, -*b*, -*c* and 'pheopigments' in Florida Bay seston with that obtained by high performance liquid chromatography-photodiode array analyses.

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ABSTRACT: While investigating pigment-based chemotaxonomy of phytoplankton in Florida Bay (Louda, 2002), a high performance liquid chromatography – photodiode array detection (HPLC-PDA) derived data on the chlorophylls (-*a*, -*b*,-c_1/-c_2) and "pheopigments" was compared to that derived from the spectrophotometric analysis of the same extracts. This comparison was prompted by the rather wide spread in data from a 1996 nine lab 'inter-laboratory comparison of chlorophyll determination' in which the author participated. The present report uses data from 244 samples of Florida Bay phytoplankton collected during monthly sampling excursions between September 2000 and June 2002. The spectrophotometric determination of chlorophyll-*a* (CHL*a*), using 5 separate published equations and 1 commercial data manipulation program, gave excellent results (y = 0.9169 – 1.0914X; R^2 = 0.9361 – 0.9987) for CHL*a*, as compared to the HPLC-PDA (X) data. The determination of "pheopigments" with the commercial program gave much better results (y= 1.0631X, R^2 = 0.463) than the classic determination using Lorenzen's (1967) equation (y= 11.178X , R^2= 0.0271), but it too was still inadequate for routine usage if conclusions on community "health" (*viz.* senescence, predation) were to be made. Comparisons of the determination of the chlorophylls-*b* or –c_1/-c_2 by spectrophotometry versus HPLC derived data proved fruitless as R_2 values were close to zero (-0.16 to 0.04) and the slope ("m" in y=mX) gave overestimations of 1.8 – 5.6. It is concluded that valid CHL*a* estimates can indeed made using spectrophotometric measures on 90% acetone extracts of Florida Bay seston (Whatman GF/F filters). However, it is also concluded that no meaningful estimates of "pheopigments" or alternate chlorophylls (-*b*, -c_1/-c_2) are possible using these methods.

This web report is a draft preprint of an article to be submitted for journal consideration, comments welcomed.

INTRODUCTION: Measurements of chlorophyll-*a* (CHL*a*), as well as other photosynthetic pigments, in the waters entering and within Florida Bay is an integral to monitoring changes which are bound to accompany the replumbing of the Everglades as the Comprehensive Everglades Restoration Plan (CERP) is enacted. In 1996, the senior author took part in a 7 laboratory-10 method interlaboratory CHL*a* determination using both unialgal cultures (3) and natural field samples (3) from Florida Bay. The intercomparison was hosted by Dr. W. Kruzinski of the US-EPA laboratory in Marathon (Vaca Key) Florida. Results of that study revealed a wide spread in resultant data. That is, the mean of spectrophotometric and fluorometric measures was about 2X the value obtained by RP-HPLC / PDA and, more troubling, the range in values

covered nearly one-half an order of magnitude (*e.g.* R = 5.2 to 26.5, x = 2.1). In that study, a single spectrophotometric and a single spectrofluorometric methods gave results quite consistent with the HPLC derived data. As there are tremendous amounts of dissolved organic matter (DOM, aka Gelbstoffe) in Florida bay waters, especially in the outflows and nearshore waters close to the mangrove transition zone, high background fluorescent signals are to be expected. Indeed, certain fluorescent background problems have been revealed (Boyer, J. 1996-8, Pers. Communs.). Thus, once the author's HPLC study (Louda, 2002) of pigment-based chemotaxonomy began, it was decided to collect all pertinent spectrophotometric data on those samples. Unfortunately, a routine filter fluorometer was not available and coincident fluorometric data are lacking.

Regarding the qualitative and quantitative analyses of microalgal pigments, there can be no doubt that the single most important text is that of Jeffrey and co-workers (1997). This volume, "*Phytoplankton pigments in oceanography"*, equally applicable to fresh waters, contains 17 chapters, a compendium of identification data, and 13 appendices. This tome was the result of an immense pigment project (WG78) under the auspices of SCOR-UNESCO. One chapter, "*Comparison between spectrophotometric, fluorometric and HPLC methods for chlorophyll analysis"* by Mantoura and colleagues (1997) is highly pertinent to the present limited study on much the same topic. However, to date, such a study on spectrophotometric chlorophyll analysis in a high DOM highly turbid estuary has yet to appear. Given that the requisite samples for such a study were being collected, it was decided to utilize these for such a study.

MATERIALS AND METHODS: Samples were collected once per month from 18 sites (see Louda, 2002) in north-central and western Florida Bay. Water was collected in 2 L brown polyethylene bottles, kept in the shade and transported to shore where they were immediately (< 3hrs. collection to freezing) filtered (Whatman GF/F) under subdued light and flash frozen in liquid nitrogen. Storage and transport of the aluminum foil wrapped quarter folded filters was on dry-ice. Pigment extraction and analyses occurred with 2 weeks of collection.

Pigments were extracted using 3 mL of 90% aqueous acetone containing a known amount of copper mesoporphyrin-IX dimethyl ester (CuMeso-IX-DME) as an internal standard (= IS). Extraction occurred with grinding in a pre-chilled (*viz.* frozen) modified Potter-Elvehjem tissue homogenizer (Kontes™ 8886000 series), sonication, steeping for 1-2 hours in a refrigerator. The extraction mix was centrifuged, decanted and the moist filter paper pellet was recentrifuged in a centrifugal filter device (Amicon Ultrafree-CL™), giving a total recovery of 93+%(2.8/3.0 mL). The pooled raw extract was then filtered through a 0.45 μm syringe filter. All procedures were at ice bath (~ 0-2^{0}C) temperature. It must be noted that modification of standard tissue homogenizers (rounded pestle) by slicing off pieces of the tip to form an irregular pointed tip, tremendously enhances the complete disruption of the GF/F filter and seston. This, with sporadic sonication (homogenizer mortar immersed into bath style sonicator), gave very good extraction. This was concluded prolonged steeping (24+hrs) brought out only minor amounts of additional pigment, 2-5% as a maximum. Potential alteration of pigments by letting them set in solvent does not warrant the minor added yield. It must be noted that this study utilized only 90% aqueous acetone as an extractant and was not designed to investigate alternate extractants (*cf.* Wright *et al*., 1997). Dimethylformamide (DMF) is reported to be superior for certain recalcitrant pigments (notably CHL*b*) but it is a strong liver toxicant which is readily absorbed through the skin and is not recommended by SCOR-UNESCO on that basis (Wright *et al*., 1997), we agree.

1.0 mL of the raw extract was taken and added to a pre-chilled vial containing 0.125 mL of an ion pairing solution (*cf.* Mantoura and Llewellyn, 1983). This mix formed the injectate and 0.10 ml (100 µL) was loaded onto the HPLC column. The HPLC conditions and gradient are given elsewhere (Louda *et al*., 1998, 2000, 2002). Pigment detection and quantitation derived from the Beer-Lambert relationship using PDA data (AU*min) and published extinction coefficients adjusted to 440 nm (chlorophylls, chlorophyllides, carotenoids), 410 nm (pheophytins, pheophorbides, pheophorbide steryl esters), or 394 nm (CuMeso-IX-DME = IS). A system response factor was applied to all pigments based on the ratio IS_{added} / $IS_{detected}$. The correction factors ranged from 1.1 – 1.3x.

The UV/Vis spectrum of an additional 1.0 mL aliquot of the filtered raw extract was recorded and instrument derived absorption values recorded at 630, 645, 647, 663, 664, 665 and 750 nm for use in the polychromatic equations to be tested. Next 1 drop of 2% HCl (w/v) was added, the solution mixed once with a Pasteur pipette and the spectrum re-recorded, this time taking absorption at 665 and 750 nm for "pheopigment" estimations.

The so-called "simultaneous equations" were taken from the literature (see references) And, along with others not tested herein, can be found in the review of Jeffrey and Welschmeyer (1997) which is Appendix F in Jeffrey *et al.* (1997*)*.

Equations tested; All results are in µg/mL, except Lorenzen (1967) and ChlCalc which give mg/m^{-3} (µg/L) directly. "A" is the absorption at the wavelength (nm) indicated by subscript:

"SCOR-UNESCO (1966)" 90% acetone;

CHL*a* = $11.64\ A_{665} - 2.16\ A_{645} + 0.10\ A_{630}$

CHL*b* = $-3.94\ A_{663} + 20.97\ A_{645} - 3.66\ A_{630}$

CHLs*c* = $-5.53\ A_{663} - 14.81\ A_{645} + 54.22\ E_{630}$

Jeffrey and Humphrey (1975) {= J&H'75 } 90% acetone;

CHL*a* = $11.85\ A_{664} - 1.54\ A_{647} - 0.08\ A_{630}$

CHL*b* = $-5.47\ A_{664} + 21.03\ A_{647} - 2.66\ A_{630}$

CHLs*c* = $-1.67\ A_{664} - 7.60\ A_{647} + 24.52\ A_{630}$

Jeffrey and Humphrey (1975) with Humphrey (1979) {= J&H'75/H'79} 90% Acetone. (chromophyte modification)

CHL*a* = $11.47\ A_{664} - 0.40\ A_{630}$

CHLs*c* = $24.36\ A_{630} - 3.73\ A_{663}$

Lorenzen (1965) Chl*a* corrected for 'pheopigments. 90% acetone.

CHL*a* = $[26.73\ (A_{665}^{o} - A_{665}^{a})v] / V$

Pheo = $[26.73\ (A_{665}^{a} - A_{665}^{o})v] / V$

Where; A_{665}^{o} and A_{665}^{a} are absorption at 665nm before and after acidification, v = volume of the pigment extract, V = volume of the water filtered, and 26.73 is an absorption coefficient correction for the ratio of these pigments with pure chlorophyll.

A commercial product "Chlorophyll Calculator™ (ver. 1.11 © 1993. SoftLabWare™, as distributed by WindowChem™, Fairfield, Ca.) was also tested.

RESULTS AND DISCUSSION:

100 to 2,000 mL, depending upon turbidity, of Florida Bay water was able to be filtered. The spectra of the extracts gave A_{664} values between 0.008 and 0.250, with a majority between 0.05 and 0.15. No attempt was made to sort results by the absorbance of the crude extract and no relation was apparent upon causal exam.

Two-hundred and forty-four samples, collected between September 2000 and May 2002, were included in this study.

Figures 1a, 1c, 1d, 2a and 3a are plots of the HPLC determined chlorophyll-*a* ($\sum$ CHL*a* = CHL*a* + CHL*a'* + CHL*a-allomer* + CHLide-*a* +pyroCHLide-*a*: namely, all CHL*a* chromophoric species) on the x-axis *versus* CHL*a* determined by the indicated spectrophotometric methodologies. It must be concluded that the correlations are all excellent in that an approximately 1:1 relationship (*viz*. slope ~ 1.0) was found with a Pearson correlation coefficient [r] also close to unity. Given that all of the CHL*a* spectrophotometric estimation trace their origins to the work of Arnon (1949) plus Richards and Thompson (1952), and the popularized revision by Parsons and Strickland (1963), with but slight alterations in the coefficients since then, the fact that all of these estimates are very close should not be too surprising. All correlations were forced through the origin (0,0), as needs to be done to maintain Beer-Lambert constraints. Resulting slopes and Pearson [r] correlation coefficients for these comparisons are also given in Table 1. It needs to stressed that, even though, the estimation of CHL*a* 'chromophores' was good-to-excellent, the inability of these methods to detect the altered chlorophylls-*a*, such as chlorophyllide-*a* or chlorophyll-*a—allomer*, does not allow any inference as to community health (*e.g.* senescence).

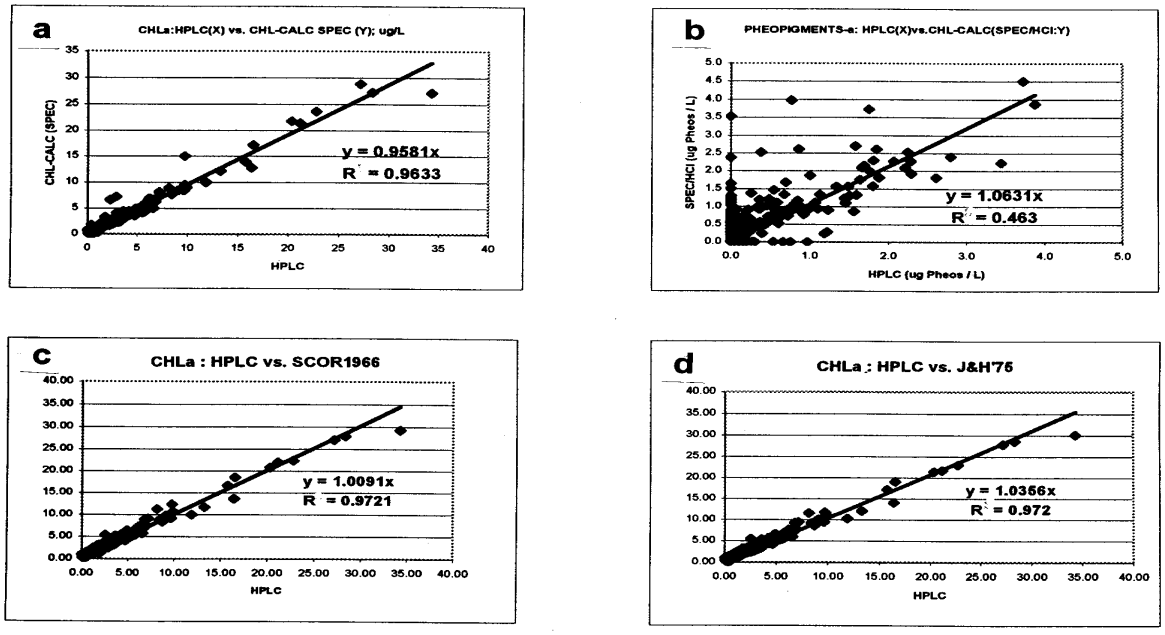

Figure 1: (a, c, d) Determination of CHL*a* by HPLC (x-axis) v. the methods of (a) ChlCalc™, (c) SCOR-UNESCO, 1966, and (d) Jeffrey and Humphrey, 1975. (b) determination of "pheopigments" by HPLC (x-axis) v. ChlCalc™.

The estimation of the "pheopigments", a term which SCOR-UNESCO WG78 does not approve of but acknowledges due to its widespread use in the literature (Jeffrey and Welschmeyer, 1997), includes measuring all of the pigments with a 'pheophorbide-*a*-like' (PHide*a*) chromophore, and therefore spectrum. This measure, if valid, can give important information as to the 'health' of a community, predation and/or to the amount of recycled / resuspended material in the seston (*cf.* Louda *et al*., 1998, 2002; Millie *et al*., 1993). However, a rapid and facile method, either by spectrophotometry or fluorometry, is apparently still lacking (see the caveats reviewed by Jeffrey and Welschmeyer, 1997). In the present case, we examined the spectrophotometric estimation of "pheopigments" in Florida Bay seston by the acidification method using the commercial ChlCalc™ software (Figure 1b) and the classic method of Lorenzen (1967: Figure 2b). The method of Lorenzen (1967) gave a slope of about 11 and essentially no correlation (r= 0.0271). However, even though the correlation coefficient of the commercial (ChlCalc™) software was poor (r=0.463), the fact that the slope was close to unity (y= 1.0631x) reveals that progress has been made since 1967. However, it must also be pointed out that, in this relationship (Figure 1b), a considerable number of samples either had "pheopigments" and were not estimated or were estimated and were not present. The only conclusion possible is that, if information on pheopigments is required, then HPLC methodology must be invoked. This is especially true if information which details predation (*viz.* pyro-pheophorbide-*a*), senescence (*viz*. pheophytin-*a*), or sediment resuspension (both) is required.

In this study, the value 'pheopigments' determined by HPLC was the sum of Pheophorbide-*a* (PHide*a*), PHidea-allomer, pyro-PHide*a*, pheophytin-*a* (PHtin*a*), PHtin-*a*' (=epimer), PHtin-*a*-allomer, pyro-PHtin*a*, PHide*a*-steryl esters, and pyroPHide*a*-steryl esters (see Louda *et al*, 2000).

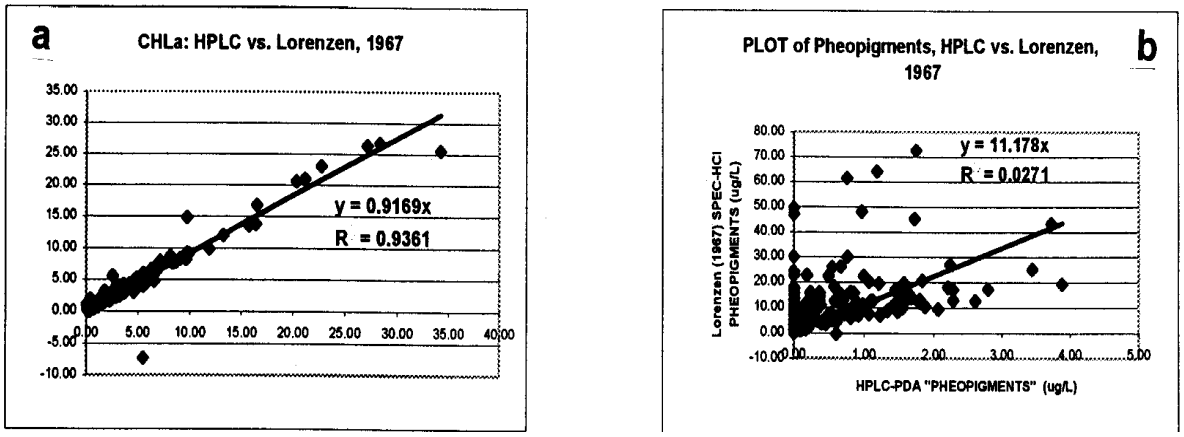

Figure 2: The 'acidification' method of Lorenzen, 1967. (a) Determination of CHL*a* v. HPLC(X-axis). (b) Determination of "pheopigments" v. HPLC (x-axis).

We also compared HPLC determinations of chlorophyll-*b* (CHL*b*: Figure3b) and the chlorophylls-*c* (=∑ CHLc_1 + *CHL*c_2 : Figures 3 c and 3d). In these cases, approximately 1.8 to 5.6 overestimations with no correlation were found. Again, at least for Florida Bay waters with their high carbonate marl and DOM load, if information on the presence and abundance of the accessory chlorophylls (-*b*, -*c*) is required, then only HPLC data will suffice.

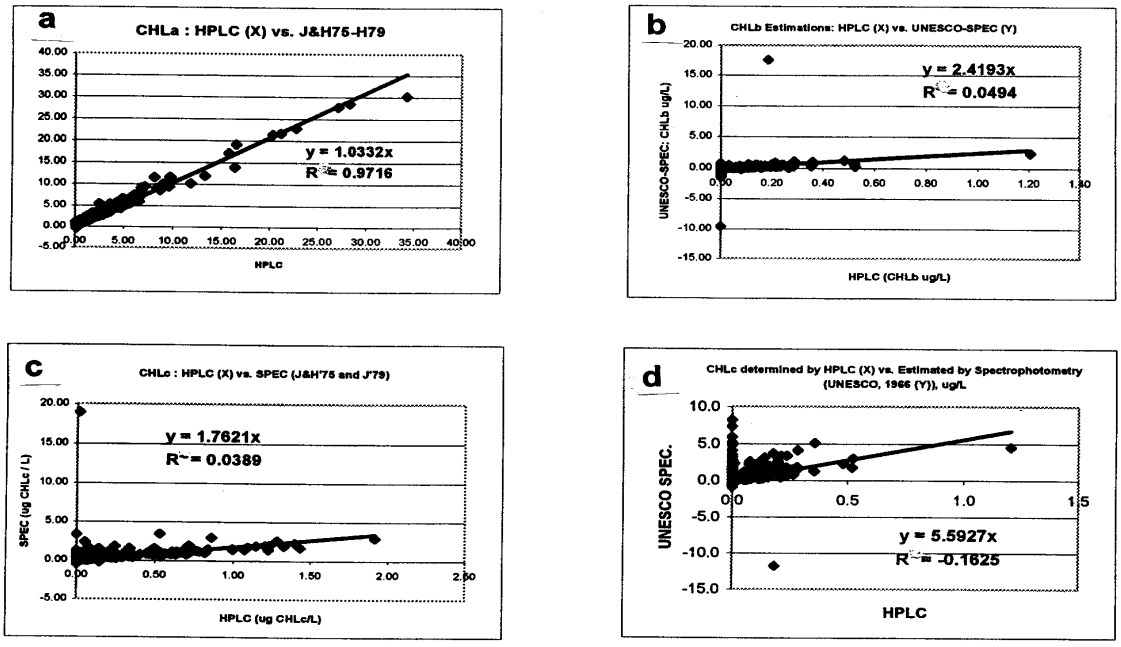

Figure 3: (a) CHL*a* determined by HPLC (x-axis) v. the method of Jeffrey and Humphrey, 1975 with Humphrey, 1979 modification. (b) CHL*b* determined by HPLC 9x-axis) v. the method of SCOR-UNESCO, 1966. (c-d) Determination of Chlorophylls-*c* by HPLC (x-axis) v. (c) the method of Jeffrey and Humphrey, 1975 with Humphrey, 1979 modification. and (d) SCOR-UNESCO, 1966.

Table 1: Compiled regression data (slope and R^2) for the comparison of chlorophylls and pheopigments in Florida Bay seston determined by HPLC (x) versus spectrophotometric (y) methodologies.

Pigment estimated		**Correlation**
METHOD (y)	**SLOPE**	**Coefficient [R^2]**
***Chlorophyll*-a**		
ChlCalc™ software	0.9581	0.9633
SCOR-UNESCO 1966	1.0091	0.9721
Jeffrey & Humphrey, 1975	1.0356	0.9720
Lorenzen, 1975	0.9169	0.9361
J&H'75 / Humphrey 1979	1.0332	0.9716

Table 1 cont.:

"Pheopigments(a)"		
ChlCalc™ software	1.0631	0.4630
Lorenzen 1975	11.178	0.0271
***Chlorophyll*-b:** SCOR-UNESCO 1966	2.4193	0.0494
***Chlorophylls*-c:** SCOR-UNESCO 1966	5.5927	-0.1625
J&H'75 with Humphrey 1979	1.7621	0.0389

Lastly, consideration of the amount of material required for a reasonable spectrophotometric CHL*a* estimate is required. That is, to quote from Jeffrey and Welschmeyer (1997):

"Ideally, enough seawater should be filtered to yield an absorbance (optical density) >0.1 at 664 nm when using the spectrophotometric acidification technique."

Examination of the rank ordered distribution of raw extract absorption values (Figure 4) obtained during the present study reveals that only 50 (20.5%) of the 244 samples analyzed met that criterion. The slopes and correlation between the spectrophotometric estimates and the HPLC derived data (Table 1) indicate, such estimates are quite good, regardless of the absolute value of the absorption of the extract.

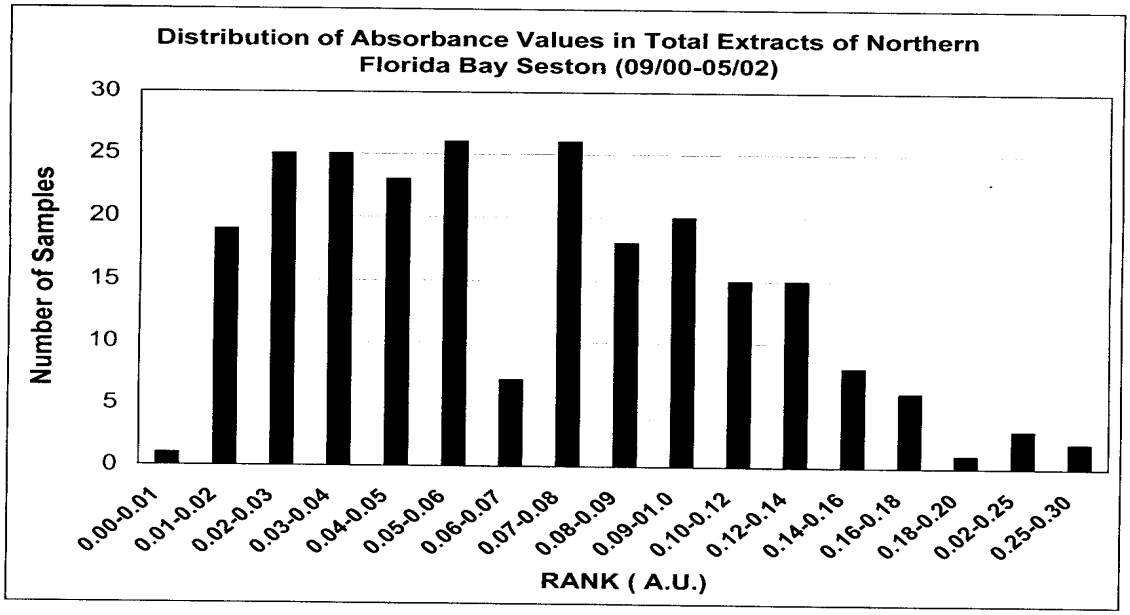

Figure 4: Rank ordered distribution of absorbance values (λ = 664 nm) for the 244 samples of northern Florida Bay seston included in this study.

Visual examination of the raw data (Excel spreadsheet available upon request) revealed that there likely was a higher degree of imprecision between the spectrophotometric estimations and the HPLC determined values when A_{664} of the raw extract was below about 0.02AU. Indeed consideration of the 20 (8.2%) samples, out of the 244, with A_{664} < 0.02AU revealed poorer

correlation coefficients (Lorenzen 1975 / r = 0.6284; SCOR-UNESCO 1966 / r = 0.7957; Jeffrey and Humphrey, 1975 / r = 0.778). However, slopes (1.0256, 1.0468, 1.0767, respectively) were still nominally at unity (3-7% overestimations). Obviously, calculation of RSDs and similar indices would allow discarding of true outliers. However, comparison of the spectrophotometric techniques with HPLC data requires inclusion of all data. That is, if only the spectrophotometric data were available, a result would not be detectable as an outlier and would be included in any data set. As spectrophotometric analyses are much less expensive and much faster than are HPLC analyses, duplicate or triplicate determinations are suggested in order to assess the validity of the spectrophotometric data. Replicate runs on our HPLC system, using the same or different extracts of the same sample, reveal very small (2-5%) variations (Louda unpubl. data; *cf.* Winfree *et al*., 1997).

The comparisons made during this study derived from water samples containing from 0.07 to 34.27 µg/L. One sample from an isolated water body well within the mangrove transition zone (Mrazek Lake, S = 9 psu) gave a total CHL*a* value of 441 µg/L and was left out of the calculations reported here. However, inclusion of the Mrazek Lake data changed the CHL*a* regressions very little (*e.g.* ChlCalc™ slope = 1.0914, r = 0.9987) but severely skewed the 'pheopigments' calculations (*e.g.* ChlCalc™ slope = 0.4166, r = -0.2109) due to the large 'lever arm' imparted by that single sample.

The rank-ordered distribution of CHL*a* concentration in the Florida Bay water samples investigated during this study is given as Figure 5. In an overly simplified manner; the low (0.7 – 2.0 µg/L) values derived from (diatom) non-bloom sequences in the north central bay, the moderate values (2-6 µg/L) came mainly from mixed phytoplankton communities (diatom, dinoflagellate, cryptophyte, chlorophyte) of the western bay, and the high values (6 – 35 µg/L) were associated with cyanobacterial bloom sequences in the north-central bay (see Louda, 2002).

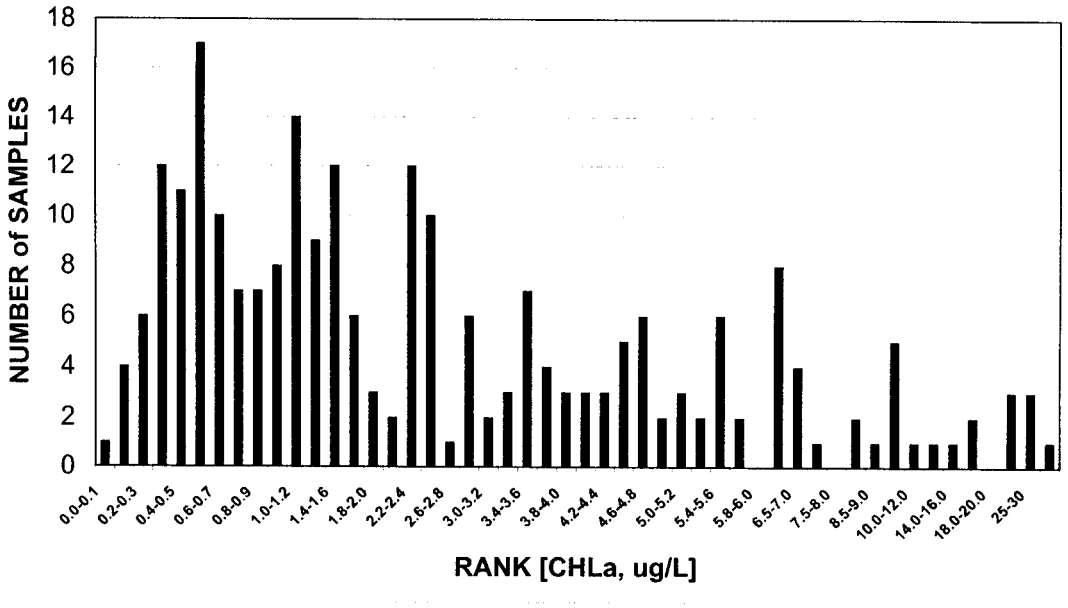

Figure 5: Rank ordered distribution of CHL*a* concentration (µg/L) in the 244 samples of north-central and western Florida Bay water analyzed from September 200 through May 2002.

CONCLUSIONS:

244 Samples of Florida Bay phytoplankton collected during monthly sampling excursions between September 2000 and June 2002 were analyzed by HPLC-PDA and spectrophotometric methods in order to determine CHL*a*, CHL*b*, CHLs-*c* and 'pheopigment' contents.
The spectrophotometric determination of chlorophyll-*a* (CHL*a*), using 5 separate published equations and 1 commercial data manipulation program, gave excellent results (y = 0.9169 – 1.0914X; R^2 = 0.9361 – 0.9987) for CHL*a*, as compared to the HPLC-PDA (X) data.

The determination of "pheopigments" with the commercial program gave much better results (y= 1.0631X, R^2 = 0.463) than the classic determination using Lorenzen's (1967) equation (y= 11.178X , R^2= 0.0271), but it too was still inadequate for routine usage if conclusions on community "health" (*viz.* senescence, predation) were to be made.

Comparisons of the determination of the chlorophylls-*b* or $-c_1/-c_2$ by spectrophotometry versus HPLC derived data proved fruitless as R^2 values were close to zero (-0.16 to 0.04) and the slope ("m" in y=mX) gave overestimations of 1.8 – 5.6.

It is concluded that valid CHL*a* estimates can indeed made using spectrophotometric measures on 90% acetone extracts of Florida Bay seston (Whatman GF/F filters). However, it is also concluded that no meaningful estimates of "pheopigments" or alternate chlorophylls (*-b*, $-c_1/-c_2$) are possible using spectrophotometric methods on these communities.

DISCLAIMER: Mention of trade names in text does not constitute an endorsement by the authors or their funding agencies (DOC, NOAA, NMFS, SFERPM). Rather, trade names were cited only to indicate a style or level of quality. Alternate suppliers for each item are available and will suffice.

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